The mouse growth hormone mRNA was isolated by RT-PCR (401) using total RNA extracted from mouse pituitary (400) and forward primer 30-1Y (5'agcgaattcgtcctgtggacagatcactgc-3'; SEQ ID NO: 5) and reverse primer 30-2Y (5'gctctcgaggaaggcacagctgctttccac-3'; SEQ ID NO:6). The 736 nt RT-PCR product was digested by EcoRl and Xhol and cloned in plasmid pCDNA1.1 (Invitrogen, Carlsbad, Ca.) that had been digested by EcoRl and Xhol (402). The resulting construct was designated VB-P1 (403). Part of the envelope proteins precursor was amplified from plasmid DH-BB (Invitrogen, Carlsbad, Ca). using forward primer 30-20V (5'cttctcgagcagtttaaacgtgagcttccc-3'; SEQ ID NO:7) and reverse primer 30-16V (5'acgtctagatcatcttcgtgtgctagtcag-3') ; SEQ ID NO:8) (404). The resulting PCR product encoded the p62 envelope protein minus the first 13 amino acids (p62d13), the 6K peptide (6K) and the E1 protein (E1). This product was digested with Xhol and Xbal and cloned in Xhol-Xbal digested plasmid VB-P1 (405). The resulting construct was designed VB173. A double-stranded oligonucleotide consisting of oligonucleotide 47-1V (5'tcgagcagatctgcagcaccactggtcacggcaatgtgtcggagcgg-3'; SEQ ID NO:9) annealed to oligonucleotide 43-2V (5'ccgctccgacacattgccgtgaccagtggtgctgcagatctgc-3'; SEQ ID NO:10) was inserted in Xhol-Pmel digested VB173 to produce VB175 (406). The latter plasmid contained the mutated p62 coding sequence (p62mutRS, i.e. arginine substituted for leucine at position 11 and serine substituted for leucine at position 12). The capsid coding sequence was amplified from DH-BB using forward primer 63-1V (5'gtgtccaagccatcagaggggaaataaagcatctctacggtggtcctaaatagtcagcatagt-3') SEQ ID NO:11) and reverse primer 28-6V (5'ccagagctcatgcggaccactcttctgt-3'; SEQ ID NO:12) (407). The forward primer contains nt -46 to +14 of the Sindbis subgenomic, +1 being the site of initiation of transcription. The reverse primer corresponds to the last 12 nt of the capsid coding sequence preceded by the sequence (5'gagctcatgcgga-3') SEQ ID NO: 33) such that the extremity of the PCR product contains, in addition to the capsid sequence, a serine codon, an alanine codon, a termination codon, and a Saci restriction site. The PCR product was cloned in the unique blunted Xbal site of VB175(408). Plasmid intermediates were constructed such that a sequence comprising the restriction sites for Notl and BamHi was inserted between the EcoRl and Xhol sites of VB188 (409). A Notl-Xhol fragment comprising nt 20-102 of the Keratin-Associated protein (KAP) was cloned in the plasmid from step 409 to produce VB-P2 (410). Insertion of this fragment encoding the first 22 amino acids of KAP will serve as a negative control since they do not encode a signal peptide. Fragments excised from VB188 and VB-P2 using Sacl and blunted using T4 DNA polymerase were cloned in the unique Pmll site of VB41 (411). This plasmid was constructed by ligating a blunted Sacl-Pmll 772 bp fragment of pSinRep5 in the

unique EcoRV site of pCDNA1.1 (both pSinRep5 and pCDNA1.1 purchased from Invitrogen. Carisbad, Ca.). The resulting plasmids contain a modified viral genome as depicted in Figure 3A and having inserted an exogenous nucleic acid encoding a signal peptide (VB192, mGH) or not (VB193, KAP). To introduce a unique restriction site after the 3' untranslated region of the Sinbis modified genome, VB193 was digested with HindII and the 5997 bp fragment was circularized to produce VB194 (412). A double-stranded oligonucleotide consisting of oligonucleotide 24-7V (5'-tcgcgatttaaattaattaagctt-3'; SEQ ID NO:13) annealed to oligonucleotide 24-8V (5'-aagcttaattaatttaaatcgcga-3'; SEQ ID NO:14) and comprising the restriction sites for NruI, SwaI, PacI, and HindIII was cloned in the unique and blunted EcoRI site of VB194 to produce VB195(413). A 4303 bp PmlI-PvuI fragment encompassing this insertion was then subcloned in a PmlI-PvuI fragment of both VB192 and VB193 to produce VB196 and VB197 respectively (depicted only for VB193). In order to be able to directionally clone cDNA fragments at the indicated BamHI site of VB197, additional steps were taken to remove the BamHI site at position 7334 relative to the first nt of the modified viral genome and sites EcoRV at positions 2748 and 6876 relative to the first nt of the modified viral genome. Removal of these sites was done by PCR-based mutagenesis according to standard protocols.

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Respectfully submitted,

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